Metaldehyde-Mediated Genotoxicity in Allium Cepa (L.) Root Meristem Cells

Oksal MACAR
Giresun Universitesi

Tuğçe Kalefetoğlu Macar (tkmacar@gmail.com)
Giresun Universitesi, Şebinkarahisar School of Applied Sciences, Department of Food Technology

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Abstract

The excessive use of metaldehyde (MA) in agriculture to combat molluscs endangers both the environment and non-target organisms. *Allium cepa* is a popular model organism for determining toxicity of commonly used chemicals such as pesticides. The aim of this study was to investigate the metaldehyde-mediated genotoxicity in *A. cepa* roots. To achieve this goal, five groups were formed and the first group considered as control was treated with tap water throughout the experiment. 20 ppm metaldehyde, 40 ppm metaldehyde, 100 ppm metaldehyde and 200 ppm metaldehyde solutions were applied to the other groups, respectively. Germination percentage, relative injury rate, root length and weight gain were analyzed to assess the effect of the pesticide on growth. Genotoxicity arisen from different doses of metaldehyde was investigated in terms of mitotic index (MI), micronucleus frequency (MN) and chromosomal aberrations (CAs). In addition, oxidative stress caused by metaldehyde was investigated by analyzing malondialdehyde (MDA) levels, as well as superoxide dismutase (SOD) and catalase (CAT) activities. The results of the growth parameters showed that metaldehyde had a growth-limiting effect in *A. cepa*, depending on the application dose. According to root elongation levels, EC_{50} (effective concentration) value for metaldehyde was 50 ppm in *A.cepa*. As the applied dose of metaldehyde increased, the incidence of MN and CAs gradually increased while MI decreased. Incrising doses of metaldehyde application also increased MDA levels, SOD and CAT activities. Data of the present study undoubtedly showed that metaldehyde had a suppressive effect in usual cell division, growth and chromosomal integrity while caused oxidative stress associated with genotoxicity.

Introduction

Pesticides are chemical compounds those enable reduction of agricultural losses, increase in yield as well as abundant and inexpensive food production. The development of pesticides, including herbicides, fungicides, insecticides and molluscicides, has increased gradually since World War II. Since then, the population boom in the 20th century has forced the increase in food production, and numerous advances in agricultural technology have been accompanied by the rise in pesticide use (Tudi et al. 2021). However, pest resistance that develops due to the increasing administration of pesticides causes both economic losses and necessitates the use of more and more toxins in fields (Schreinemachers and Tipraqsa 2012). While only 1% of the 3 million tonnes of pesticides used in the world each year are effective to protect target crops from pests, the rest accumulate in the environment and cause health problems for non-target species. (Bernardes et al. 2015; Hayes et al. 2017).

Molluscs such as slugs and snails are perilous pests not only for crops, but also to a wide variety of agricultural products including vegetables, ornamental plants, paddy and oilseeds, especially in the rainy seasons (Zhang et al. 2011). Metaldehyde ($C_8H_{16}O_4$ = 2, 4, 6, 8 - tetramethyl - 1, 3, 5, 7 - tetraoxacyclooctane) is a molluscicide that has been practiced to exterminate gastropods since the 1940's (Castle et al. 2017). As a polar dry alcohol, it is produced by polymerization of acetaldehyde. Once taken into the body, it causes the molluscs to secrete excessive mucus and dry out completely (Triebskorn et al. 1998). This chemical compound and residues of which could be found in harvested fruits and

vegetables, is capable of entering the bloodstream through digestion in humans and other non-target creatures, causing poisoning (Moreau et al. 2015). According to the studies focused on mammals, pets and wild animals, metaldehyde has been classified as a moderately toxic compound (Saad et al. 2017). It also has neurotoxic effects and causes vomiting, tachycardia, tachypnea, ataxia, tremors and seizures that can result in death (Dolder 2003). Concerns about metaldehyde pollution are increasing due to its very long half-life (water; 17 and soil; 223 d) and low biodegradability (Dong et al. 2017).

Allium cepa L. has become one of the most used model organisms in plant-based toxicity studies due to its large sized and small number of chromosomes that can be easily seen under light microscopy, easy accessibility, low cost, reliability and high correlation with other test systems (Kalefetoğlu Macar et al. 2021; Pantano et al. 2021). *Allium* assay has been used for many years to elucidate the genotoxic effects of pesticides in living organisms (CherMacar 2020; Sheikh et al. 2020).

Although the toxic effects of metaldehyde have been studied before, studies on its genotoxic potential on plants are scarce. The aim of the study was to assess genotoxic effects of metaldehyde molluscicide by evaluating MI and the frequencies of MN and CAs besides growth parameters in *A. cepa* model plant.

Materials And Methods

Preparation of materials and experimental setup

A. cepa bulbs purchased from a local grocery store were selected to be approximately equal in weight (7.10-9.00 g). The bulbs were thoroughly washed under running tap water to remove dust. The brown scales on the outermost part of the bulbs were peeled off and the old roots were cut away. *Allium* bulbs were then divided into five groups consisting of a control and four treatment groups. There were 50 bulbs in each group. The control group was kept in glass tubes filled with tap water so that the basal plate of the bulbs touched water throughout the experimental process. Treatment groups 1, 2, 3 and 4 were exposed to aqueous solutions of 20 ppm metaldehyde, 40 ppm metaldehyde, 100 ppm metaldehyde and 200 ppm metaldehyde solutions, respectively. All applications were carried out in a dark chamber at room temperature for 72 hours.

Analysis of growth parameters

Once the experiment was terminated, root elongation was assessed measuring the lengths of the adventitious roots those grow during the experiment by a ruler. The EC_{50} value, the point indicating 50% of the growth, was determined using the root length measurement of five different groups. The bulbs were weighed at the end of the experiments. To determine the weight gain, the difference between the final weight and the weight recorded before the experiments was taken for each bulb. The emergence of adventitious roots from the basal plate of the bulbs was considered as “germination” to calculate the germination percentage (Atik et al. 2007): (Eq.1). Relative injury rate (RIR) was calculated using the formula (Eq.2)

GP (%) = (Number of the germinated bulbs / Total number of the bulbs) x 100 (Eq.1).

RIR= (%GP in control group - %GP in each group) / (%GP in control) (Eq.2).

Analysis of genotoxicity parameters

The roots were decapitated to perform the analysis of cytogenetic parameters. The frequencies of both CAs and MN incidences were determined according to the method of Staykova et al. (2005). Root tips were fixed using Clarke's fixator (glacial acetic acid / ethanol =3:1) and washed thoroughly with distilled water. Root tips were hydrolyzed at 60°C using 1 N hydrochloric acid for 12 min. Hydrolyzed root tips were washed again with distilled water before stained with 1% acetocarmine for 24 hours at room temperature. To prepare examination slides, root tips were squashed between slide and cover slip with a drop of 45% acetic acid solution. Ten slides from each treatment were observed under a research microscope at 500X magnification. The method of Fenech et al. (2003) used to evaluate MN frequency. CAs and MN frequencies were calculated by examining 100 cells from each slide (1,000 cells for each treatment). On the other hand, MI was determined by examining 100 cells from each slide (10,000 cells for each treatment). MI was calculated as the ratio of cells in the mitotic phase to the total number of cells observed.

Analysis of SOD and CAT activities

Analysis of SOD and CAT activities were performed using the same extraction method. 0.2 g root sample was homogenized in 5 mL cold 50 mM sodium phosphate buffer (Ph 7.8) using cold pestle and pestle. After the homogenate was centrifuged at 10,000 rpm for 20 minutes, the supernatant was used to determine the SOD and CAT activities.

The method proposed by Beauchamp and Fridovich (1971) was used to evaluate activity SOD enzyme. SOD enzyme activity was determined by measuring the reduction of nitro blue tetrazolium (NBT) spectrophotometrically at 560 nm. Results of SOD enzyme activity were expressed as units per milligram fresh weight (Unit / mg fresh weight).

The method mentioned by Beers and Sizer (1952) was used to evaluate activity CAT enzyme.

CAT enzyme activity was determined by measuring the enzymatic breakdown of H_2O_2 spectrophotometrically at 240 nm. Results of SOD enzyme activity were expressed as OD_{240} nm min /g fresh weight.

Analysis of MDA levels

At the end of the 72th hours, MDA levels of A. cepa roots samples of groups were analyzed using the method proposed by Unyayar et al. (2006). 0.5 gr root sample was homogenized in 5% trichloroacetic acid (TCA) solution with a mortar and pestle. Obtained homogenates were centrifuged at 12,000 rpm for 14 min at room temperature. Supernatant and 20% TCA–0.5% thiobarbituric acid (TBA) solution were

mixed in the same amounts in a test tube. Test tube with mixture was heated in a hot water bath at 98 °C for 23 min in a hot water bath. At the beginning of 24th min, the test tube was put in an ice bath to stop the reaction. Cooled mixtures were centrifuged at 10,000 rpm for 5 min at room temperature. Supernatant was taken and its absorbance at 532 nm and 600 nm measured using a spectrophotometer (Shimadzu 1240 UV–Vis spectrophotometer)

Results And Discussion

Growth analyses enabled us to evaluate the macroscopic effects of different metaldehyde doses in *A. cepa* (Table 1). While the germination percentage of the control group was 100%, the germination percentage decreased as the metaldehyde dose increased in the metaldehyde applied groups. Therefore, the most prominent drop in the germination percentage of the treatment groups was observed in MA-200 ppm. Treatment 1, exposed to a lower metaldehyde concentration, had a lower relative injury rate (0.06). Relative injury rates of MA-20 ppm, MA-100 ppm and MA-200 ppm were 0.16, 0.30 and 0.42, respectively. Metaldehyde also inhibited the root growth of the groups depending on the application dose. Root elongation was reduced by 23% in MA-20 ppm and 72% in MA-200 ppm compared to control. The EC_{50} value is a useful parameter for selecting test concentrations to perform genotoxicity tests (Chauhan et al. 1986). In this study, EC_{50} value for metaldehyde on *A. cepa* was determined as 50 ppm. This result confirms that the concentrations selected in the study are suitable for genotoxicity and toxicity tests. Metaldehyde-related deceleration of weight gain was statistically significant in all groups, similar to inhibition of root elongation. Weight gain decreased by 16%, 26%, 40% and 60%, respectively, from the group treated with the lowest dose of metaldehyde (MA-20 ppm) to the group treated with the highest dose (MA-20 ppm), compared to the control. Although there are many studies in the literature on the toxicity of metaldehyde in non-target organisms such as ducklings, dogs, cats and macroinvertebrates (Wei et al. 2020; Botelho et al. 2020; Bergamini et al. 2020; Gething et al. 2020), to the best of our knowledge this is the first study to reveal metaldehyde toxicity in *A. cepa*. On the other hand, Ester and Nijenstein (1996) mentioned that metaldehyde application to perennial ryegrass (*Lolium perenne*) at rates exceeding 320 g per kg seed had a phytoxic effect by reducing germination. Roots are the main gateways for the entrance of a metaldehyde to a plant during germination (Simms et al. 2006). Therefore, it is not surprising that the first place where chemical damage to the plant can be morphologically observed is the roots. The reductions of root elongation and weight increase suggest that the rate of mitosis in meristematic tissues was suppressed by metaldehyde.

Table 1. Effects of metaldehyde treatments on growth parameters.

*Groups	Germination percentage (%)(n=50)	Relative injury rate	Root length (*cm*) (n=10)	Weight gain (*g*) (n=10)
MA-0 ppm (Control)	100	0.00	8.56±1.17[a]	2.44±0.10[a]
MA-20 ppm	94	0.06	6.55±1.91[b]	2.05±0.07[b]
MA-40 ppm	84	0.16	4.78±1.21[c]	1.80±0.08[c]
MA-100 ppm	70	0.30	3.82±2.42[d]	1.46±0.09[d]
MA-200 ppm	58	0.42	2.38±0.55[e]	0.97±0.10[e]

*The means shown with different letters (a-e) in the same column were significant at p <0.05.

In order to determine the genotoxic effects of metaldehyde on *A. cepa* root meristem cells, the MI values and frequencies of MN and CAs were investigated (Table 2) (Figure 1). MI provide valuable information about the toxic and genotoxic effects of chemicals as an indicator of cell proliferation rate. Increasing metaldehyde doses decreased the MI values of MA-20 ppm group (24%), MA-40 ppm group (37%), MA-100 ppm (48%) and MA-200 ppm group (57%). Application of metaldehyde reduced the successful mitosis rate during germination process. Results of MI were well-correlated with our growth parameter, particularly with decreases in root elongation and weight gain. Similarly, Asita and Hatane (2012) reported that application of mixture of metaldehyde, 30 g/kg and Carbaryl, 20 g/kg on *A.cepa* reduced the MI value. The determination of MN has a very important role in investigating the toxicity and genotoxicity of pesticides (Karaismailoglu 2017). Contrary to MI values, MN frequencies on *A. cepa* meristem cells increased gradually as a result of metaldehyde treatment in dose dependent manner (Figure 1a). Among the metaldehyde treatments, the highest MN frequency was observed in the MA-200 ppm treatment (45.50±2.90) and the lowest MN frequency was observed in the MA-200 ppm treatment (17.90±2.69). MN formation may be the result of breaks in microtubules and chromosomes or single-strand breaks in DNA (Konuk et al. 2007; Qureshi et al. 2016). Although no previous report was found about metaldehyde-induced MN formation, many researchers considered increasing MN frequencies as a sign on genotoxic effect of pesticides in plants (de Souza et al. 2017; Datta et al. 2018; Kalefetoğlu Macar 2020; Macar 2020). Parallel to the MN frequencies, frequencies of all CA types were also gradually increased by increasing doses of metaldehyde applications. Frequencies of CAs caused by metaldehyde were sorted from high to low as follows; sticky chromosome (Figure 1b), vagrant chromosome (Figure 1c), fragments (Figure 1d), unequal distribution of chromatin (Figure 1e), reverse polarization (Figure 1f), bridge (Figure 1g) and multipolar anaphase (Figure 1h). In accordance with our study, Asita and Hatane (2012) reported that sticky chromosomes were most seen CAs as a result of metaldehyde/carbaryl mixture application. Sticky chromosomes were most possible CA formation in the case of deterioration in DNA (Mercykutty and Stephen 1980). Sticky chromosomes can result from the adhesion of chromosomal proteins or defects nucleic acid metabolism in the cell, or the dissolution of the protein that covers the DNA (Sheikh et al. 2020). On the other hand, vagrant and laggard chromosomes are indicators of spindle malfunction

while fragments and bridges are attributed to clastogenicity (Rank 2003). Considering that bridge and fragment aberrations are directly related to MN formation (Bianchi et al. 2016), the increases of these aberrations were paralleled the results of MN in this study. Drastic increases in the frequencies MN and CAs induced by metaldehyde clearly demonstrated genotoxic properties of metaldehyde. These increased MN and CAs also caused a retardation in MI and growth parameters by reducing the rate of successful cell division in mitosis phase.

Table 2. Genotoxic effects of metaldehyde on *A. cepa* meristematic root cells.

*Damage	Control	MA-20 ppm	MA-40 ppm	MA-100 ppm	MA-200 ppm
MI	872 ± 29,13^{a}	661.60±36,60^{b}	553.10±34.67^{c}	456.80±22.74^{d}	377.60±28.79^{e}
MN	0.38±0.29^{e}	17.90±2.69^{d}	26.30±2.90^{c}	35.90±2.60^{b}	45.50±2.90^{a}
SC	0.14±0.09^{e}	27.70±0.79^{d}	44.40±2.55^{c}	56.20±3.85^{b}	73.30±5.14^{a}
VC	0.40±0.33^{e}	20.70±2.79^{d}	28.40±2.07^{c}	37.10±3.28^{b}	46.70±2.36^{a}
FRG	0.00±0.00^{e}	15.50±2.55^{d}	23.50±2.01^{c}	30.50±2.42^{b}	39.50±2.76^{a}
UDC	0.00±0.00^{e}	7.90±2.49^{d}	14.50±1.78^{c}	20.20±2.35^{b}	29.20±2.15^{a}
RP	0.00±0.00^{e}	4.50±2.72^{d}	11.30±2.83^{c}	19.10±2.02^{b}	26.60±2.07^{a}
B	0.00±0.00^{e}	3.70±2.11^{d}	9.20±1.48^{c}	16.10±2.47^{b}	22.90±2.13^{a}
MA	0.00±0.00^{e}	2.10±1.45^{d}	7.20±1.75^{d}	13.90±2.60^{b}	18.10±1.79^{a}

*Data were shown as mean ± SD. MI: mitotic index, MN: micronucleus, SC: sticky chromosome, VC: vagrant chromosome, FRG: fragments, UDC: unequal distribution of chromatin, RP: reverse polarization, B: bridge, MA: multipolar anaphase. The averages shown with different letters (a-d) in the same line are statistically significant ($p<0.05$).

Metaldehyde-induced changes in MDA level and SOD and CAT activities are shown in Table 3. Metaldehyde application gradually increased MDA level and activities of SOD and CAT enzymes. The highest dose (200 ppm) of metaldehyde caused the highest statistical increase in all biochemical parameters when compared to those in control ($p < 0.05$). In the MA-200 ppm group, MDA level and activities of SOD and CAT enzymes were increased by metaldehyde more than twice of their control group counterparts. Toxic compounds can affect the activities of antioxidant enzymes such as SOD and CAT, which indicate the both toxicity level and tolerance capacity of plants (Kaya and Doganlar 2016). CAT and SOD enzymes are important parts of plants antioxidant system, and their increased levels were indicators of elevated level of reactive oxygen species (ROS) in *A. cepa* due to pesticides (Sivakumar et al. 2010; Macar 2020). Similarly, excessive ROS production increases membrane lipid peroxidation in plants, which can be measured by MDA content (Srivastava and Sing, 2020). In the present study, the

gradual increase in MDA level indicated increased membrane damage caused by metaldehyde-induced ROS accumulation. Similarly, increased CAT and SOD enzymes due to the plant's activated oxidative defence system indicated an increased ROS level caused by metaldehyde. Increased level of ROS can induce serious harmful effects including severe DNA damage (Nan et al. 2016). In this context, our biochemical parameters showing ROS accumulation are compatible with genotoxic and growth parameters. Biochemical parameters of our study revealed that metaldehyde caused oxidative stress which triggered in heavy cell membrane damages and genotoxic injuries. The antioxidant defence system containing SOD and CAT enzymes was activated against metaldehyde, but could not eliminate all of its undesirable effects.

Table 3. Biochemical effects caused by metaldehyde.

Groups*	SOD (U / mg FW)	CAT (OD_{240nm}min / g FW)	MDA (µmol / g FW)
MA-0 ppm (Control)	71.637 ± 5.20^{e}	0.54 ± 0.07^{e}	7.21 ± 0.36^{e}
MA-20 ppm	96.07 ± 3.78^{d}	0.76 ± 0.04^{d}	9.97 ± 0.47^{d}
MA-40 ppm	127.05 ± 2.05^{c}	0.94 ± 0.03^{c}	11.97 ± 0.50^{c}
MA-100 ppm	139.30 ± 3.44^{b}	1.10 ± 0.04^{b}	14.13 ± 0.55^{b}
MA-200 ppm	151.47 ± 5.27^{a}	1.24 ± 0.05^{a}	16.33 ± 0.47^{a}

* The averages shown with different letters$^{(a\text{-}d)}$ in the same column are statistically significant ($p<0.05$).

Conclusion

Metaldehyde has been widely used as a successful molluscicide, but there is insufficient information about its effects on non-target organisms. *A. cepa* is a well-known and accomplished model plant for genotoxicity studies. A versatile research procedure including growth, genotoxicity and biochemical parameters was carried out to reveal the phytotoxic and genotoxic effects of metaldehyde on *A. cepa*.

It has been concluded that the application of metaldehyde caused growth retardation, genotoxicity and oxidative stress on *A. cepa* in a dose dependent manner. Metaldehyde exposure negatively affected germination and growth process of *A. cepa*. EC_{50} value for metaldehyde on *A. cepa* was determined as 50 ppm. Reduced MI and increased MN and CAs levels clearly indicated a metaldehyde-induced genotoxicity. Metaldehyde triggered oxidative damage and promote ROS production, acting as a genotoxicity enhancer. There are studies in the literature that present different opinions on the phytotoxicity of metaldehyde in plants. However, this study clearly demonstrated that this pesticide showed genotoxic and phytotoxic effects on *A. cepa* assay, which was highly correlated with mammals. The results of this

study revealed the necessity of new and detailed studies on the undesirable effects of metaldehyde on non-target organisms, including humans.

Declarations

Ethical Approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

Authors' contributions Dr. Oksal Macar and Dr. Tuğçe Kalefetoğlu Macar, carried out the experimental stages, manuscript preparation and statistical analysis.

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Competing interests The authors declare that they have no competing interests.

Data availability All data generated or analyzed during this study are included in this published article.

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Figures

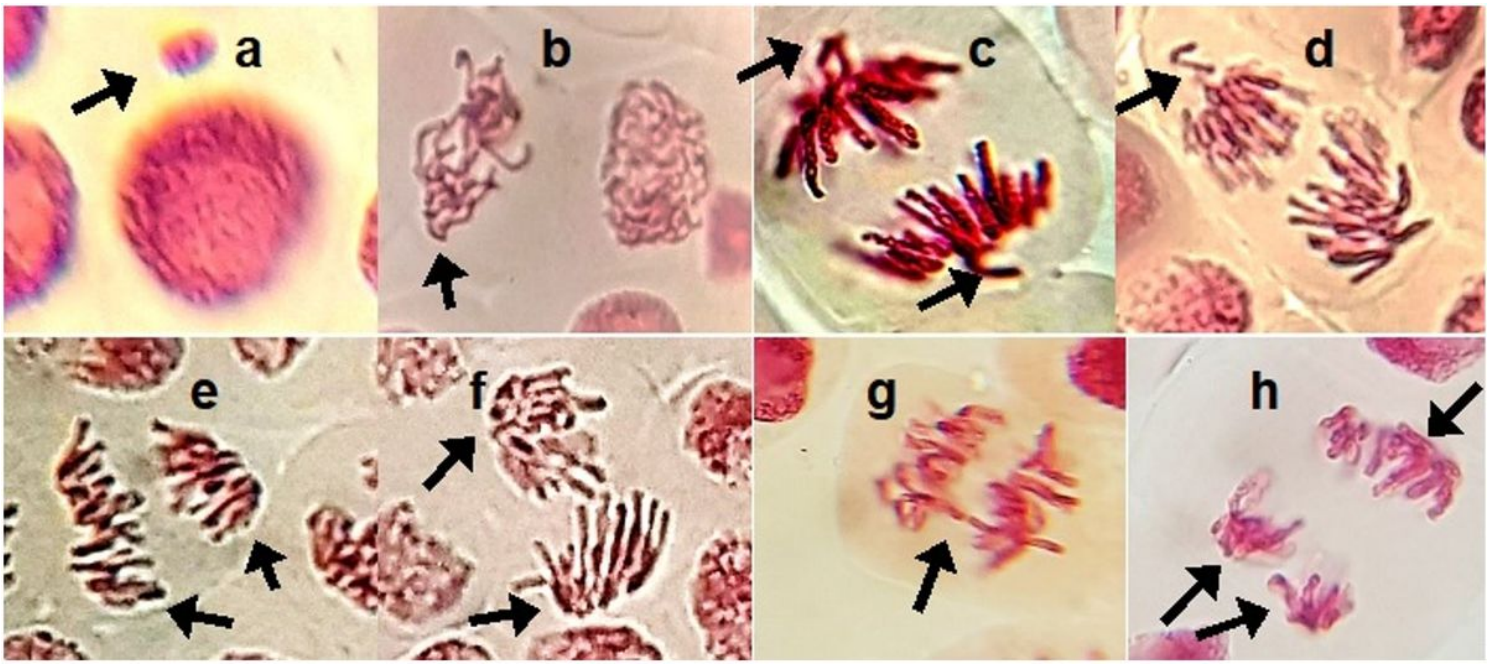

Figure 1

Metaldehyde induced chromosome aberrations. (a; Micronucleus, b; Sticky chromosome, c; Vagrant chromosome, d; Fragment, e; Unequal distribution of chromatin f; Reverse polarization, g; Bridge h; Multipolar anaphase)